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ORIGINAL ARTICLE

The role of pp38 in regulation of Marek's disease virus bi-directional promoter between pp38 and 1.8-kb mRNA

Jiabo Ding · Zhizhong Cui · Lucy F. Lee · Xiaoping Cui · Sanjay M. Reddy

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Abstract Marek's disease virus (MDV) contains a bi-directional promoters located between pp38 gene and 1.8-kb mRNA in the long inverted repeat region of the viral genome. The involvement of pp38 gene in up-regulating the activity of these promoters was analyzed by transient expression of chloramphenicol acetyltransferase (CAT) reporter gene. Two CAT reporter plasmids, pP(pp38)-CAT and pP(1.8-kb)-CAT, were constructed to express CAT under the control of the bi-directional promoter in both orientations. These plasmids were transfected into chicken embryonic fibroblast (CEF), infected with rMd5 and pp38 deleted rMd5 (rMd5/Δpp38), respectively. No CAT activity was detected in uninfected CEF as expected. CAT activities in rMd5/Δpp38 virus infected CEF (rMd5/Δpp38-CEF) were 3.5-fold lower using pP(pp38)-CAT and 12-fold lower using pP(1.8-kb)-CAT than those of the parental rMd5 infected CEF (rMd5-CEF). The significantly lower promoter activity in the pp38 deletion virus suggests that pp38 can regulate the activity of the bi-directional promoters, especially in the direction of 1.8-kb mRNA family. Co-transfection of pp38-expressing plasmid (pcDNA-pp38) into rMd5/Δpp38-CEF significantly increased the activity of the bi-directional promoters using either pP(pp38)-CAT or pP(1.8-kb)-CAT. DNA mobility shift assay showed a binding of the 73-bp sequence of the bi-directional promoter with rMd5-CEF but not with rMd5/Δpp38-CEF or uninfected CEF lysates. However, rMd5/Δpp38-CEF lysates could bind the same 73-bp promoter sequence when co-transfected with pp38-expressing plasmid (pcDNA-pp38). All these data taken together suggest pp38 plays an important role in regulating the transcriptional activity of the bi-directional promoter.

Keywords Marek's disease virus (MDV) · pp38 · Bi-directional promoter · Chloramphenicol acetyltransferase (CAT) · DNA mobility shift

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Introduction

Marek's disease virus (MDV) is an oncogenic herpesvirus, which causes a highly contagious neoplastic disease in chickens, Marek's disease (MD) [1]. Marek's disease is characterized by the development of T-cell lymphomas and lymphocytic infiltration of nerves and other organs. The disease can be prevented by vaccination with all the serotypes of MDV [2]. Serotype 1 MDV is the prototype virus and consisting of oncogenic strains and their attenuated viruses. Two additional serotypes consisted of nononcogenic herpesviruses isolated from chickens (serotype 2) and turkeys (serotype 3). All the serotypes of MDV share similar genomic organization, significant DNA homology and antigenic cross reactivity [3–7]. The lymphomagenesis of MDV has been studied in its pathology, virology, and immunology. However, the molecular basis for neoplastic transformation of lymphocytes by MDV still needs to be elucidated. Four of the serotype 1 MDV genes have been

reported to be related to tumorigenesis: the 1.8-kb mRNA transcript with 132-bp repeats [8, 9], the 38 kd phosphorylated protein gene (*pp38*) [10, 11], the oncogene, M*eq* [12], and the immediate early ''gene, ICP4 [11]. One of the MDV'' unique genes, pp38, is located at the junction of the internal repeat long and the unique long region of the MDV genome. It is highly conserved among strains of serotype 1 MDV and a homolog of pp38 exists in both serotypes 2 and 3 MDV but with significantly different sequences. Serotype 1 MDV pp38 is encoded by an unspliced message of 1.9-kb and localizes primarily to the cytoplasm of MDV-infected and MD induced tumor cells. Since pp38 was the only antigen detected in MD tumors or MDV-transformed cell lines for over a decade, and since it shares the promoter–enhancer of the 1.8-kb gene family, it was thought to be a protein involved in transformation. Recently, by inoculation of MDV-susceptible birds with the *pp38* deletion mutants virus, it is reported that pp38 was involved in early cytolytic infection in lymphocytes but not in the induction of tumor [13].

The 1.8-kb mRNA transcript is present only in oncogenic serotype 1 viruses but is truncated in attenuated variants [14, 15], this transcript is mapped in the internal repeat long region of the MDV genome containing the tandem repeats of the 132-bp repeat region. This repeat region is expanded during in vitro passage and the 1.8-kb mRNA becomes disrupted and heterogeneous [9]. In virulent oncogenic strains of MDV, there are only two copies of the 132-bp but are multiple copies up to 100 in attenuated viruses [16, 17]. For a very long time, it was suggested that the expansion of the 132-bp region, disrupts the 1.8-kb mRNA that is essential for oncogenicity, thereby resulting in attenuation [9, 18]. Recently, Silva et al. [19] showed that deletion mutants of 132-bp region still remained oncogenic. Therefore, the function of 1.8-kb mRNA in MDV is still not clear.

Located between pp38 and 1.8-kb mRNA in the repeated long region of the MDV genome contains a short fragment of a bi-directional transcriptional promoter sequence that controls the transcription of both genes in opposite orientations. Although the promoter sequence is only 305 bp in size, it contains the replication origin and several *cis*-acting motifs such as TATA-box, CAAT-box, Oct-1, and Sp1 [8, 10, 20], In the middle of this promoter region, there is a 90-bp replication origin of MDV genome [8, 21] which shares more than 80% nucleotide identity among the serotypes of MDV, and over 70% identity with those of other α-herpesviruses [22]. It was reported that when the bi-directional promoter was inserted into plasmids with chloramphenicol acetyltransferase (CAT) reporter gene, it was expressed transiently only in MDV-infected CEF but not in normal CEF, indicating that there must be a viral or cellular factor(s) involved in regulation [23]. In this paper, using a pair of recombinant MDVs, rMd5 and its pp38 deleted rMd5 virus (rMd5/Δpp38), we report that pp38 is one of the viral factors which influences the activity of the bi-directional promoter.

Materials and methods

Cells and viruses

Primary chicken embryonic fibroblasts (CEF) were used for virus propagation and DNA transfections. A pair of molecularly engineered recombinant MDVs, rMd5 and rMd5/Δpp38 were used in this study. The difference between these two viruses is that the pp38 gene present in rMd5 but deleted in rMd5/Δpp38 [13].

Construction of recombinant plasmids expressing CAT gene under the control of the bi-directional promoter

The bi-directional promoter sequences were amplified by PCR. For the promoter P(pp38) in pp38 transcriptional direction with forward primer: 5′-GCGA***GGTACC***AGAGAGCATCGCGAAGAG-3′ (bases −693 to −676, relative to pp38 gene ORF, plus a *Kpn*I linker); and reverse primer: 5′-CCTGA***GAGCTC***TTATCCTATACCG-3′ (bases −325 to −337 plus a *Sac*I linker). For the promoter P(1.8-kb) in the 1.8-kb mRNA transcript direction, the forward primer: 5′-CCTGA***GGTACC***TTATCCTATACCG-3′ (bases −325 to −337, plus a *Kpn*I linker), and reverse primer: 5′-GCG***GAGCTC***AGAGAGCATCGCGAAGAG-3′ (bases −693 to −676, plus a *Sac*I linker). The PCR product of 369-bp contained the whole promoter–enhancer of 305-bp [23]. The PCR products were inserted into pCAT-Basic vector (Promega) at the *Kpn*I and *Sac*I sites. In the recombinant plasmids, pP(pp38)-CAT and pP(1.8-kb)-CAT, CAT was expressed under the regulation of the promoter in opposite directions. The construction diagram is shown in Fig. 1.

To construct the recombinant plasmid pcDNA-pp38, pp38 gene (bases 1–873) was amplified with primers: 5′-AAT***GGATCC***ACTCATGACCCACATGGAA-3′ as forward primer (bases −13 to 6, plus a *Bam*HI linker); and 5′-CGAG***CTGCAG***ATCGGGTACGGCTACACTG-3′ as the reverse primer (bases 900–882, plus a *Pst*I linker). PCR product was cloned into pcDNA-3.1/Zeo(+) vector (Invitrogen) at the *Bam*HI and *Sal*I site. All the plasmid DNAs were purified with the Qiagen kit (Qiagen) and the concentration of the DNA was determined prior to transfection.

Transfection of pP(pp38)-CAT and pP(1.8-kb)-CAT to uninfected CEF, rMd5-CEF and rMd5/Δpp38-CEF

Primary CEF cultures were prepared in 60 cm^2 flask until cells formed a monolayer and infected with rMd5- or rMd5/Δpp38-CEF stocks of about 1×10^5 plaque form unit (pfu).

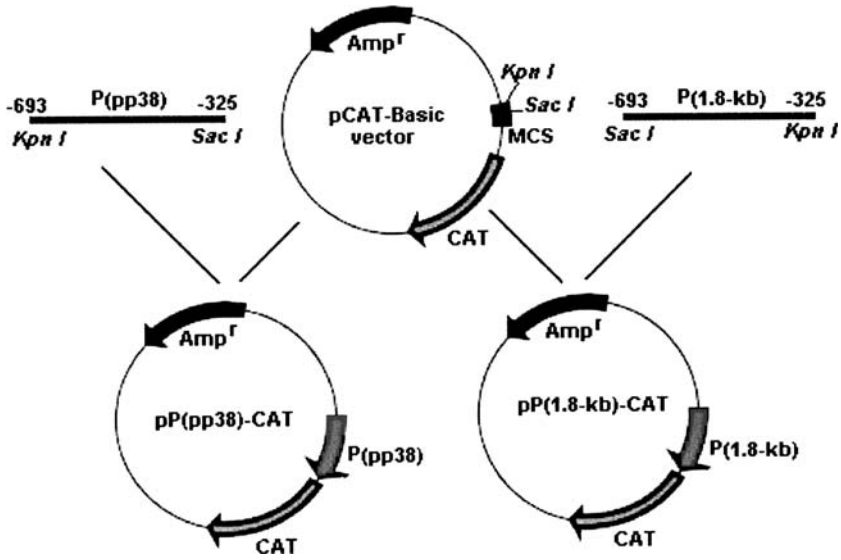

Fig. 1 Construction of recombinant plasmids expressing CAT gene under the control of the bi-directional promoter

The infected cell cultures were incubated for 3–4 days until cytopathogenic effect (CPE) was formed in about a quarter of cells in the monolayers. The MDV-CEF monolayers were trypsinized and the viable cell number was determined. One part of the MDV-CEF suspension was mixed with two parts (by cell number) of fresh secondary CEF suspension and placed into 35 mm dishes (1×10^6 cells per dish). To prepare the secondary CEF monolayers, 1×10^6 cells were seeded into 35 mm dishes until cell monolayers formed 18–24 h later.

Transfection was carried out 18 h later when the secondary CEF monolayers were formed. Transfection of recombinant plasmid DNA was performed by using LipofectAMINETM reagent (Gibco BRL) according to the manufacturer's instructions. Briefly, 2 μg plasmid DNA and 4 μl LipofectAMINETM reagent (Gibco BRL) were added into two separated polypropylene tubes with 100 μl of DMEM medium free of serum and antibiotic. These two solutions were mixed and incubated for 45 min at room temperature and then added into another 800 μl DMEM. A total of 1 ml of the transfection solution was carefully poured onto the cell monolayers in a 35 mm dish. After 8 h, 1 ml of complete medium with 10% bovine fetus serum were added to the transfected cell monolayers. All dishes were maintained at 37°C in a CO_2 incubator. The expression of CAT was determined 48 h after transfection. When pcDNA-pp38 was cotransfected with pP(pp38)-CAT or pP(1.8-kb)-CAT, 2 μg plasmid DNA each was mixed with 4 μl LipofectAMINETM reagent for transfection in a 35 mm dish.

Determination of CAT activity in transfected CEF, rMd5-CEF and rMd5/Δpp38-CEF cells

Two days after transfection with plasmids pCAT-Basic (control), pP(pp38)-CAT and pP(1.8-kb)-CAT, the transfected CEF were harvested and resuspended in 500 μl lysis buffer (0.25 M Tris–HCl, pH=7.0) per 35 mm dish. After 3 freeze-thaw cycles, samples were centrifuged for 5 min at 10,000 rpm. Aliquots (200 μl) of the supernatants were added into wells of 96-well ELISA plates to test CAT activity using CAT ELISA Kit (Roche, Cat.No.1363727). The concentration of the CAT in the lysates was measured using a calibration curve of known specific standards according to the manufacturer's instructions. Five replicates of transfections were carried out with three different CAT plasmid DNAs in each of rMd5-CEF or rMd5/Δpp38-CEF or uninfected CEF cells. The significant differences among the groups were analyzed by student's test. CAT activity in the co-transfected samples were also determined and analyzed as described.

Detection of pp38 in transfected CEF with indirect fluorescence antibody test (IFA)

IFA with anti-pp38 specific mouse serum (Cui, unpublished data) was used to detect the expression of pp38 in pcDNA-pp38 plasmid DNA transfected CEF or rMd5/Δpp38-infected CEF monolayers on coverslips harvested at 24, 48 and 72 h after transfection. The cell monolayers were fixed with cold acetone:alcohol mixture (2:1), and 50 μl of 1:500 dilution of anti-pp38 mouse serum was added and incubated for 45 min at 37°C. After washing 3 times with 1× PBS (pH=7.2), 50 μl of 1:256 dilution of anti-mouse IgG sheep serum conjugated with FITC (Sigma) was added and incubated for 1 h at 37°C. After washing 3 times, the coverslips were mounted and observed under a fluorescence microscope. Unimmunized mouse serum was used as a negative control.

Preparation of cytoplasmic extracts for DNA-binding assay

Normal CEF and rMd5/Δpp38-CEF monolayers in 60 mm dishes were transfected with plasmids pcDNA-pp38, respectively. Forty-eight hours after transfection, the culture medium was removed and washed 3 times with PBS. The transfected cells were scraped into PBS in a 1.5 ml tube and centrifuged at 1850×*g* for 5 min. The packaged cell pellets were re-suspended into 5 times (v/v) of hypotonic buffer [10 mM HEPES (pH=7.9), 15 mM $MgC1_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT] and centrifuged for another 5 min at 1850×*g*. They were re-suspended in 3 times (v/v) of the hypotonic buffer as the original packed cell pellet and cells were swelled on ice for 10 min or more till the lysis was more than 80% complete. Next, the lysis was centrifuged for 15 min at 3300×*g* and the supernatant was collected. After adding 0.11 volume of 10× cytoplasmic buffer [0.3 M HEPES (pH=7.9), 1.4 M KCl, 0.03 M $MgCl_2$] in the saved supernatant, they were centrifuged for 1 h at 100,000×*g*. Supernatants were dialyzed for 2 h against 50 volume of dialysis buffer [20 mM HEPES, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT]. After dialysis, the cytoplasmic extract was centrifuged for 20 min at 25,000×*g*. The supernatant was aliquoted into sample tubes of 40 μl each and stored at −80°C for use. In addition, the untransfected normal CEF, rMd5-CEF or rMd5/Δpp38-CEF monolayers were also treated as above.

Preparation of Dig-labeled DNA probes for mobility shift assay

The single stranded DNA fragments representing 3 subregions I, II and III (67, 73 and 58-bp) of the bi-directional promoter sequence (Fig. 2) were synthesized by commercial service (Bioasia company, Shanghai). At the same time, 3 primers of 10-bp complement to 3′-end of subregions I, II and II were also synthesized. The Dig-labeled double stranded DNA probes corresponding to subregions I, II and III were prepared by Digoxigenin DNA Labeling and detection Kit (Roche, Cat. No. 1093657) as following: The 3 single stranded DNAs were used as templates, and 3 complementary strands were synthesized with their own primers (as above) by klenow enzyme in Digoxigenin-labeling dNTP mixture. The detail is according to the manufacturer's instruction. The final Dig-labeled DNA probe of 20 μl was from labeling reactions with 1 μg of original synthesized single stranded DNA templates.

Mobility shift DNA-binding and detection

In a microcentrifuge tube, a 40 μl cytoplasmic extract prepared from different CEF monolayer as above was

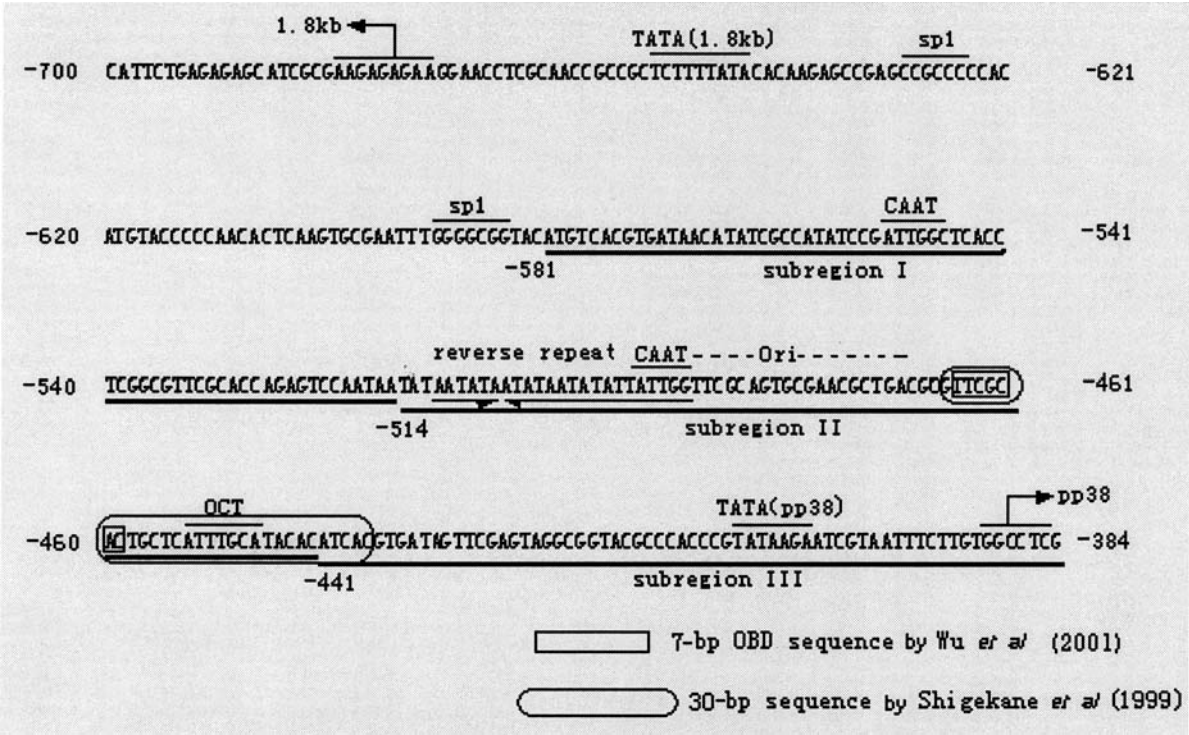

Fig. 2 DNA sequence shows the structure of the bi-directional promoter and locations of the 3 subregions used for DIG labeling DNA probes. The sequence was according to the previous publication (Cui et al. [11])

incubated in a 20 μl working solution: 2 μl Digoxigenin-labeled DNA probes (relative to subregions I, II or III), 18 μl of 300 μg/ml BSA in distilled water and incubate at 30°C for 15 min. These samples were then applied to a nondenaturing 4% polyacrylamide gel and electrophoresis was carried out. The gel was transferred to a nitrocellulose membrane (NC). The free probe or protein-bound probe DNA on the NC was detected by immunological reactions with anti-Digoxigenin-AP conjugate and then NBT/BCIP as substrate solution according to the manufacture's handbook of the Digoxingenin DNA labeling and Detection Kit (Roche, Cat. No.1093657). To identify the specific protein of pp38 in the retarded DNA complex, monoclonal antibody (Mab) H19 [24] specific to pp38 was used in Western blotting according to published procedure [13].

Result

Comparisons of CAT expression level in rMd5 and rMd5/Δpp38 viruses

To analyze the regulation activity of the bi-directional promoter for CAT reporter gene expression, plasmids pP (pp38)-CAT and pP (1.8-kb)-CAT with the promoter in opposite directions were used to transfect CEF monolayers infected with rMd5, rMd5/Δpp38 or uninfected CEF. The results indicated that CAT activity was at the base line level in uninfected CEF but at higher levels in rMd5-CEF or rMd5/Δpp38-CEF transfected with CAT reporter plasmids. The CAT expression level in rMd5-CEF was 3.5-fold higher than that of rMd5/Δpp38-CEF when transfected with plasmid pP (pp38)-CAT (52±6.28 vs. 15±2.8, $P<0.01$). The CAT activity was 12-fold higher in rMd5-CEF than that in rMd5/Δpp38-CEF when transfected with the plasmid pP(1.8-kb)-CAT (781±55.1 vs. 65±8.22, $P<0.01$) (Table 1). This result indicates that factors present in infected cells in addition to pp38 significantly affected the activity of this promoter–enhancer.

Co-transfection with pp38-expressing plasmid pcDNA-pp38 increased CAT expression levels in rMd5/Δpp38-CEF under the control of the promoter in both directions

To determine whether pcDNA-pp38 plasmid has abundant expression of pp38 protein in CEF, we used indirect fluorescence antibody test (IFA) with anti-pp38 mouse serum (Fig. 3) and western blot with monoclonal antibody H19 to study its expression (Fig. 4). As shown, pp38 is clearly expressed in CEF transfected with pcDNA-pp38 plasmid (IFA), or co-transfected with pcDNA-pp38 and different CAT reporter plasmids (Western blot), or in rMd5/Δpp38-CEF co-transfection with pcDNA-pp38 and pP(1.8-kb)-CAT plasmids. The CAT activity was 1.5-fold higher when co-transfected with pP(pp38)-CAT or 3.3-fold higher when co-transfected with pP(1.8-kb)-CAT than that in rMd5/Δpp38-CEF without pp38 expression, respectively (Table 2, $P<0.01$). All experiments in Tables 1 and 2 were done at the same time, they were separately presented in two tables for ease of interpretation. The influence of pp38 on the promoter for 1.8-kb mRNA transcript was significantly higher than that for the direction of pp38. For uninfected CEF, co-transfection of the pcDNA-pp38 with either of the two CAT plasmids did not increase the activity when compared to that in mock with pCAT-Basic.

Comparison of the activity of the bi-directional promoter in two opposite directions

The activities between the two directions of the promoter were demonstrated quantitatively when the CAT reporter plasmids were used. As indicated in Table 1, CAT activity in rMd5-CEF transfected with pP(1.8-kb)-CAT was about 15-fold higher than that transfected with pP(pp38)-CAT (781±55.1 vs. 52±6.28, $P<0.01$). Even in rMd5/Δpp38-CEF, reporter plasmid pP(1.8-kb)-CAT induced 4.3-fold higher than that of pP(pp38)-CAT (65±8.22 vs.15±2, $P<0.01$). The CAT activity in rMd5/Δpp38-CEF transfected with pP(pp38)-CAT was only slightly but still sig-

Table 1 Comparison of CAT activity in CEF, rMd/Δpp38-CEF or rMd5-CEF transfected with CAT reporter plasmids*

Transfected CEF	pCAT-Basic (control)	pP(pp38)-CAT	pP(1.8-kb)-CAT
Uninfected CEF	3±0 (3–3)*	4±0 (4–4)	4±0 (4–4)
rMd5/Δpp38-CEF	3±0 (3–3)	15±2.8 (11–17)	65±8.22 (53–76)
rMd5-CEF	3±0 (3–3)	52±6.28 (41–60)	781±55.1 (704–842)

*The numerical number represents Mean ± SE of five replicate assays with a given reporter plasmid. The CAT activity was compared for each pairs related to factors such as CEF infection status and the direction of the bi-directional promoter. Under the control of the promoter for pp38 direction, the CAT activity in rMd5-infected CEF was nearly 3.5-fold higher than that of rMd/Δpp38-infected CEF ($P<0.01$). Under the control of the promoter for 1.8-kb direction, the CAT activity in rMd5-infected CEF was 15-fold higher than that of rMd/Δpp38-infected CEF ($P<0.01$). The CAT activity in rMd5/Δpp38-infected CEF transfected with pP(pp38)-CAT is significantly higher than that in CEF transfected with pP(pp38)-CAT or in rMd5/Δpp38-infected CEF transfected with pCAT-Basic ($P<0.05$)

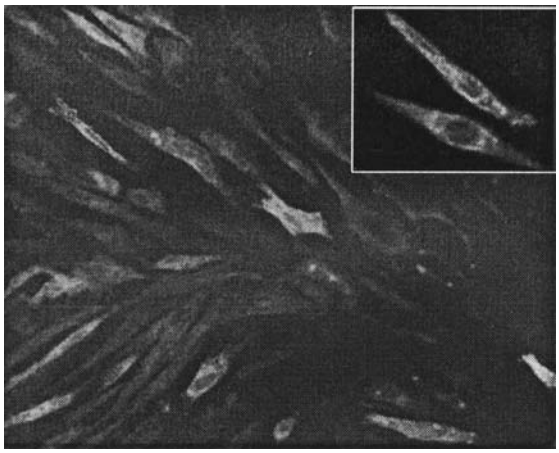

Fig. 3 Detection of pp38 by IFA with anti-pp38 mouse serum in CEF transfected with reporter plasmid pcDNA-pp38. The fluorescence was located in cytoplasm. Original magnification ×200. (Cells in up-right corner, ×400)

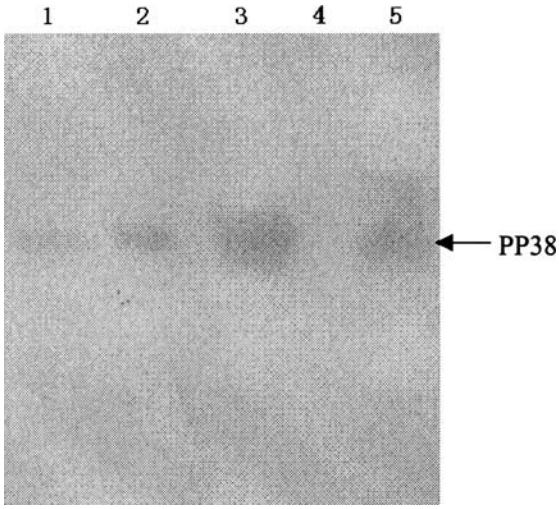

Fig. 4 Demonstration of pp38 expression in cell lysates by Western blot with pp38-specific monoclonal antibody H19. Samples were from transfection assays for Tables 1 and 2 and kept at −70°C before Western blot. (1) Lysates of CEF co-transfected with pcDNA-pp38 and pCAT-basic; (2) lysates of CEF co-transfected with pcDNA-pp38 and pP (pp38)-CAT; (3) lysates of CEF co-transfected with pcDNA-pp38 and pP(1.8 kb)-CAT; (4) lysates of rMd5Δpp38-CEF transfected with only pP(1.8 kb)-CAT; (5) lysates of rMd5Δpp38-CEF co-transfected with pcDNA-pp38 and pP(1.8 kb)-CAT

nificantly higher ($P < 0.05$) than that of mock plasmid pCAT-Basic without any promoter. However, no CAT activity was detected in uninfected CEF transfected with all the reporter plasmids (Table 1).

Determination of pp38-bound fragments of the bi-directional promoter by DNA mobility shift assays

Digoxigenin-labeled DNA fragments corresponding to 3 subregions of the promoter were prepared and tested in DNA mobility shift assays (Fig. 2). The labeled DNA fragments were treated with cellular extracts of rMd5-CEF, rMd5/Δpp38-CEF transfected with or without pcDNA-pp38, and uninfected CEF transfected with or without pcDNA-pp38. Only the subregion II of the 73-bp from base −514 to −442 relative to the pp38 ORF was retarded in gel electrophoresis by the cellular extracts from rMd5-CEF and from rMd5\Δpp38-CEF transfected with pcDNA-pp38 (Figs. 5, 6a). The cellular extracts from rMd5/Δpp38-CEF without pcDNA-pp38 transfection or from uninfected CEF did not show any mobility shift. In addition, the cellular extracts from uninfected CEF transfected with pcDNA-pp38 also did not show any mobility shift. These results suggest that the subregion II of 73-bp in the bi-directional promoter is the binding site for pp38. The other two subregion DNA fragments (67 and 57-bp) sequences (relative to bases −581 to −515 and bases −441 to −384) did not bind pp38 under the same experimental conditions in the DNA mobility shift assay (data not shown), indicating they are not the binding site for pp38.

Demonstration of pp38 in the retarded DNA complex

To confirm that the subregion II of 73-bp in the promoter was bound by pp38, the DNA mobility shift assay was conducted in duplicate according to the conditions described in Fig. 5. After transfer of non-denatured gel to nitrocellulose paper, one piece was used for demonstration of retarded DNA fragments (Fig. 6a), while the other piece was treated with pp38-specific Mab H19 by Western blotting. The pp38 was shown in the forms of both free molecules and retarded DNA/pp (Fig. 6b, lanes 2 and 4).

Discussion

The pp38 gene is the first unique MDV gene identified and also the first gene deleted from MDV genome for elucidation of the biological function in its host [10, 13]. As an early gene, the protein coded by pp38 was the first gene product detected early in MDV-infected cells or MDV-transformed cell line cells [25]. It is abundantly expressed in lytically infected cells [26]. Previous studies by Schat et al. showed that over expression of pp38 in MDV-infected REV-transformed cell line cells depressed the cytotoxic lymphocyte responses [27, 28]. Baigent et al. [29] reported that the susceptibility of chickens to MD was associated with greater numbers of $pp38^+$ lymphocytes in line 7_2 chickens. We previously published that the recombinant pp38 expressed in baculovirus infected Sf9 cells showed immunosuppressive effects in chickens to mouse red blood

Table 2 Comparison of CAT activity in CEF and rMd/Δpp38-CE co-transfected with pcDNA-pp38 and different CAT reporter plasmids*

Cells	Transfected with different report plasmids				
	No promoter	Promoter for pp38 orientation		Promoter for 1.8-kb mRNA orientaion	
	pCAT-Basic	pP(pp38)-CAT	pP(pp38)-CAT + pcDNA-pp38	pP(1.8-kb)-CAT	pP(1.8-kb)-CAT + pcDNA-pp38
Uninfected CEF	3±0 (3–3)**	4±0 (4–4)	3±0 (3–3)	4±0 (4–4)	3±0 (3–3)
rMd5/Δpp38-CEF	3±0 (3–3)	15±2.8 (11–17)	23±4.2 (19–27)	65±8.22 (53–76)	216±24.1 (189–254)
Ratios	1.00	3.75	7.67	16.25	72.00

*All experiments in Tables 1 and 2 were done at the same time, the data were separately presented in two tables for ease of interpretation. The data for rMd5/Δpp38-CEF transfected with pP(pp38)-CAT and pP(1.8-kb)-CAT were used repeatedly in both Tables 1 and 2

**The numerical figures represent following data: mean ± SE of 5 repeated transfection assays with a given reporter plasmid

Co-transfection with pcDNA-pp38 and CAT-reporter plasmids significantly increased CAT activity when compared with that in CEF transfected with CAT-expressing plasmids only under the control of both directions of the promoter ($P < 0.01$)

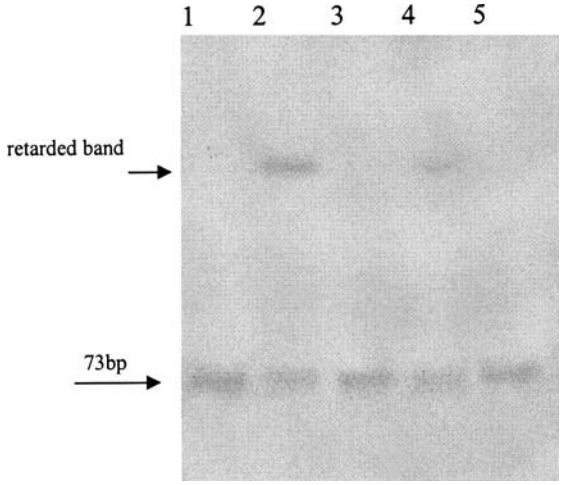

Fig. 5 Gel mobility shift assay of Dig-labeled subregion II DNA fragment of 73-bp. (1) The cellular extract of CEF transfected with pcDNA-pp38; (2) the cellular extract of rMd5-CEF; (3) the cellular extract of rMd5/Δpp38-CEF; (4) the cellular extract of rMd5/Δpp38-CEF transfected with pcDNA-pp38; (5) the cellular extract of normal CEF. The retarded bands were only demonstrated in lane 2 and 4. But the original free Dig-labeled subregion II fragments of 73-bp were demonstrated in all 5 lanes

cells [30]. Recently, Reddy et al. [13] reported the generation of a deletion mutant virus lacking the unique MDV gene pp38 (rMd5/Δpp38). They studied the in vitro and in vivo characteristics of this mutant virus and found that while pp38 is not essential for replication in vitro, the deletion virus was severely impaired for its ability to replicate in lymphoid organs. Although rMd5/Δpp38 was severely impaired for in vivo replication, the virus retained a low level of oncogenicity and suggested that pp38 was dispensable for tumor induction. This study conclusively demonstrated that pp38 was involved in early cytolytic infection in lymphocytes of MDV infected chickens, but not in the induction of tumor.

It has been recognized for many years that there is a bi-directional promoter of about 300-bp between the transcription start sites of the pp38 and 1.8-kb mRNA transcripts genes [8, 10, 21]. It was reported that CAT activity under the control of the bi-directional promoter was only detected in MDV-infected CEF but not in uninfected CEF when transfected with CAT reporter plasmids, indicating that the bi-directional promoter requires either viral or MDV-infection related cellular factors for regulation [23]. It is not known which viral product may be involved in transactivating this promoter. To demonstrate whether pp38 gene plays such role on the bi-directional promoter, the pp38 deletion virus rMd5/Δpp38-CEF was used in this study. It was shown that the CAT expression activity in rMd5/Δpp38-CEF was significantly lower than that in rMd5-CEF transfected with two CAT reporter plasmids under the control of the bi-directional promoter in two opposite orientations. To further confirm whether pp38 plays as a factor in regulating the activity of the bi-directional promoter as M*eq* is transactivating its own promoter [31], co-transfections of different CEF monolayers with CAT-reporter plasmid and pp38-expressing plasmid were conducted and compared. Co-transfection of normal CEF with CAT reporter plasmids and pp38-expressing plasmid pcDNA-pp38 did not change the CAT expression level. However, the CAT activities were significantly increased bi-directionally with additional expression of pp38 gene in rMd5/Δpp38 infected CEF cells. The CAT activities for the 1.8 kb mRNA was significantly higher than that for pp38. The data also showed that expression of pp38 by pcDNA-pp38 increased CAT activity 3-fold in rMd5/Δpp38-CEF transfected with pP(1.8-kb)-CAT, it still did not fully reconstitute the activity in rMd5-CEF. We therefore conclude that pp38 is a critical factor in regulating this bi-directional promoter. Although other factors in addition to pp38 in MDV infection may also be involved in regulating the promoter.

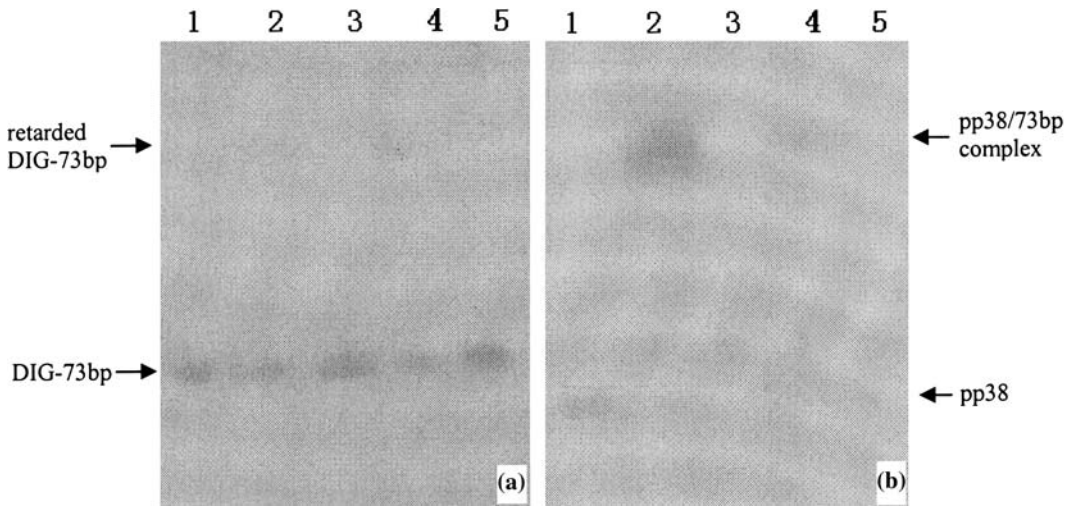

Fig. 6 Demonstration of pp38 in the retarded DNA complex. The DNA mobility shift assays in duplicate were conducted in non-denatured gels and then blotted to two pieces of NC papers. Anti-Dig conjugate was used in panel **a** for detection of free or retarded Dig-labeled 73 bp DNA fragments; in panel **b**, pp38 protein was detected by Western blot in one or two forms: pp38 free molecule (lower band), and retarded bands as DNA/pp38 complex (upper band). Figure 6a was similar to Fig. 5, but conducted in separated experiments. For both **a** and **b**, Dig-labeled 73 bp DNA fragment were treated with: (1) cellular extract of CEF transfected with pcDNA-pp38; (2) cellular extract of rMd5-CEF; (3) cellular extract of rMd5/Δpp38-CEF; (4) cellular extract of rMd5/Δpp38-CEF transfected with pcDNA-pp38; (5) cellular extract of uninfected CEF

To determine if pp38 could bind to the promoter, we used three Digoxigenin-labeled probes which overlap the entire promoter regions assayed by gel mobility shift assay. We found only the 73-bp double stranded DNA fragment was retarded at the same position by cellular extracts from rMd5-CEF and rMd5/Δpp38-CEF transfected with pcDNA-pp38. On the other hand, no mobility shift was detected with the cellular extracts from rMd5/Δpp38-CEF without pcDNA-pp38. Demonstration of pp38 in the retarded DNA complex in Western blot in non-denatured condition (Fig. 6b) indicates that the pp38 was a partner in DNA binding and the binding site resides within the 73-bp located in subregion II. The cellular extract from normal CEF transfected with pcDNA-pp38 showed no mobility shift, suggesting that co-existing or interaction with other MDV-infection related factor(s) is necessary for pp38 to bind the promoter sequence. Shigekane et al. [23] reported that the bi-directional promoter activity in two opposite orientations was regulated by a viral or cellular factor(s) induced by MDV infection, and such factor(s) bind to a 30-bp fragment in the promoter region [23]. The 30-bp fragment identified in their study is within the 73-bp fragment identified in this study. It is possible that pp38 is one of the factor(s) they mentioned but not yet identified as a viral factor. Based on the data presented in this report, pp38 is conclusively shown to play an important role in regulating the transcriptional activity of the bi-directional promoter; it is especially significant in up-regulating the 1.8-kb mRNA The binding site is located within the 73-bp of the subregion II of the bi-directional promoter.

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